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Toll-like receptors 2 and 4, and acute phase cytokine gene expression in dexamethasone and growth hormone treated dairy calves

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Abstract

Cattle are exposed to growth hormone stimulants and to stressors that cause cortisol release. Both of these hormones affect immune responses which may reduce disease resistance. Toll-like receptors are the pattern recognition molecules of pathogens that are on immune cells. They then orchestrate the induction of the appropriate acute phase cytokines of the early innate response. The objective of this study was to determine changes in toll-like receptors and acute phase cytokines following treatment with a synthetic glucocorticoid (dexamethasone) and growth hormone (GH). Twenty-eight calves were given the control (Cnt), dexamethasone (DEX), GH, or dexamethasone and GH (Both) treatments from 3 until 56 days of age. Blood was collected by jugular venipuncture on days 14, 28, 42, and 56. On day 56, a lung lavage was performed and spleen and thymus tissues collected. Total RNA was extracted from blood leukocytes, lung lavage cells, spleen and thymus cells. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was used to quantify interleukin-1 (IL-1), IL-1 receptor antagonist (IL-1Ra), tumor necrosis factor (TNF)-α, toll-like receptor 2 (TLR2), and toll-like receptor 4 (TLR4). Blood leukocytes had a time effect for IL-1Ra ($P < 0.01$), with a trend for a treatment effect ($P = 0.07$) and had a treatment by time interaction ($P < 0.05$). IL-1, TNF, and TLR2 and TLR4 were greatest ($P < 0.05$) for Cnt only at day 14. IL-1 expression of lung lavage cells was greatest ($P < 0.05$) for calves on the Both treatment compared to the other three treatments. However, IL-1Ra was not different among the treatments. Toll-like receptor 2 expression was enhanced with Both compared to either DEX ($P < 0.05$) or GH ($P < 0.05$) and tended to be greater than Cnt expression ($P = 0.07$). Expression of TLR4 tended to be reduced by Both compared to Cnt ($P = 0.06$). Tumor necrosis factor-α was greatly enhanced by Both compared to the other three treatments ($P < 0.05$). Spleen cell tended to have different IL-1 expression between GH and Both ($P = 0.07$). Interleukin-1 receptor antagonist and TLR2 and TLR4 were not different among treatments. However, TNF-α expression was enhanced by the DEX treatment alone compared to the GH treatment ($P < 0.05$), and tended ($P < 0.10$) to be greater than Cnt expression. None of the gene expressions were different among treatments for thymus cells. Lung lavage cell expression appears to be most susceptible to these hormones while blood leukocyte expression was only slightly affected, and thymus cells were not affected at all. These data demonstrate that TLR2 and TLR4 and acute phase cytokine expression can be altered by stress and growth hormones, which may decrease resistance of those animals to disease.

Keywords: Cattle; Acute phase; Cytokines; Interleukins; TNF; Real-time PCR

Abbreviations: TLR, toll-like receptor(s); Cnt, control; DEX, dexamethasone; GH, growth hormone; RT, reverse transcriptase

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1. Introduction

Toll-like receptors are part of the IL-1 family. Their function is to distinguish antigens and to initiate an appropriate immune response (Jefferies and O'Neill, 2002; Medzhitov, 2001; Modlin, 2002; Dobrovolskaia and Vogel, 2002; Takeuchi and Akira, 2002). To date, 10 TLRs have been identified (Zarember and Godowski, 2002). Although there is some overlap, each has a unique function in antigen detection. Zymosan, heat shock protein (HSP60), and gram positive bacteria are recognized by TLR2 which exists as a heterodimer, needing an association with TLR1 or TLR6 to signal. In contrast TLR4 exists as a homodimer and is responsible for gram negative lipopolysaccharide (LPS) recognition and cell-signaling. Each leads to the expression of distinct inflammatory genes (Supajatura et al., 2002). Toll-like receptor 4 leads to increased interferon (IFN)-β, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-13, RANTES, IP-10 but not IL-4 nor IL-5. Toll-like receptor 2 leads to increased IL-4, IL-6, IL-8, IL-13, IL-5, and IL-10, but not IL-1β. Other studies (Carl et al., 2002) show that induction of sIL-1Ra gene is regulated differently by the TLR2 and TLR4 agonists, LPS and peptidoglycan (PGN). Many of the cytokines that TLR2 and TLR4 induce have been studied in cattle (Burton et al., 2001; Leutenegger et al., 2000). Acute phase cytokines in bovine innate neutrophil immunity include TNF-α, IL-1, and platelet activating factor (McClenahan et al., 2000). Interleukin-1 is necessary for release of IL-8 (essential for neutrophil recruitment) by bovine epithelial cells in airways and intestinal tissues. Boudjellab et al. (2000) demonstrated that epithelial cells play an important role in immunoregulation, by this cytokine induction. The cell signals that follow binding of the pathogen to TLR and the associated MyD88 or MAL (O'Neill, 2002), induce interleukin-1 receptor-associated kinase (IRAK, an active kinase) or IRAK-M (an inactive kinase), initiating the cascade leading to cytokine induction and ultimate pathogen containment. The inactive kinase, Irak-M, is believed to regulate TLR signaling and innate immune homeostasis (Kobayashi et al., 2002).

Most tissues express at least one TLR, while several tissues express all of the known TLR (spleen and peripheral blood leukocytes). Professional phagocytes express the greatest variety of TLR. Interestingly, some TLRs are more restricted to B-cells, suggesting intrinsic roles of TLRs for adaptive immunity (Zarember and Godowski, 2002). Toll-like receptor 2, but little TLR4 is present on human neutrophils (Kurt-Jones et al., 2002). However, Sabroe et al. (2002) showed that monocytes directed previously observed leukocyte LPS response patterns of neutrophils. Monocytes, macrophages, and dendritic cells of cattle contain mRNA for boTLR2 and boTLR4 (Werling and Jungi, 2003). However, no differences in mRNA transcript quantity was detected between these TLR.

The expression of TLR have recently been studied with a variety of pathogens. The published research not only explores the TLR and pathogen interaction, but also demonstrates that a number of other variables in the physiological milieu can alter the outcome. An overlapping of pathways seems to exist between growth hormone and IL-6, but the synergistic effect of glucocorticoids are limited to growth hormone (Paul et al., 2000; von Laue et al., 2000). Glucocorticoids synergistically enhanced influenza induced TLR2 up-regulation (Shuto et al., 2002). However, TLR4 was necessary for a robust cytokine response to *Haemophilus influenza* by pulmonary epithelial cells, which lead to increased cytokine concentrations in lung lavage fluids (Wang et al., 2002).

There is evidence that species differences exist in TLR activity. Species specificity was shown in actions induced by *Salmonella* lipid A when human M-2 and TLR4 were substituted in mice (Murio et al., 2002). Very little is known of the responses of these receptors during infection in farm species, although the literature is exploding with new data using mice and human cell lines as subjects (Heldwein and Fenton, 2002; Takeuchi and Akira, 2002; Shuto et al., 2002). With the advent of limiting antibiotic use except for therapy and the use of novel immune modulators, an understanding of the function and dysfunction of toll-like receptors is needed, particularly in the context of infection status relative to necessary stressors of livestock. Many neonatal dairy calves are transported within the first week of life. Determining the sensitivity of the TLR and acute phase cytokines following stressors may help in evaluating stress effects on neonatal immune development. Innate immune functions are the primary line of defense for neonatal calves. Acute phase cytokines that predominate during

a response are IL-1, IL-6 and TNF-α. Since there is overlap in IL-1 and IL-6 functions, we focused on IL-1, its receptor antagonist and TNF. Our hypothesis was that tissues with resident macrophages will have more TLR2 and TLR4 expression than tissues such as the spleen containing more lymphocytes. We also expect that the blood monocytes will vary in TLR expression with age of the calf as more pathogens are encountered in the environment. We believe that TLR and acute phase cytokine expression will be suppressed by glucocorticoids. Therefore, our objective was to assess the expression of two toll-like receptors (2 and 4) and acute phase cytokines from various tissues following dexamethasone and growth hormone treatment.

2. Materials and methods

Twenty-eight Holstein bull-calves that were purchased from one source (a local dairy) and that had received 3 days of colostrum, were blocked by age to within 3 days. They were then fed milk replacer twice daily and offered a grain-based dry feed ad libitum. Treatments were randomly assigned within blocks ($n = 7$ calves per treatment) and initiated upon arrival from the local dairy, approximately a 1 h transport. Treatments were the control (Cnt), dexamethasone (DEX), GH, or dexamethasone and GH (Both) and were given from 3 until 56 days of age.

The control calves were fed colostrum on the first 3 days and milk replacer up to 56 days of age and received a daily injection of saline. The DEX treated calves were fed as Cnt calves and received dexamethasone (30 μg/kg body weight per day; according to Coxam et al., 1990) injected twice daily, once before each feeding. The growth hormone group was fed as the Cnt group and were given GH as Posilac on day 7 and every 14 days thereafter. The group that received Both were fed as Cnt, but also received both dexamethasone (30 μg/kg body weight per day) and growth hormone (as above).

Blood was collected by jugular venipuncture on days 14, 28, 42, and 56. Calves were exsanguinated on day 56, a lung lavage was performed (Eicher et al., 1994) and spleen and thymus tissues collected and placed immediately into RNA later (Ambion, Austin, TX). Total blood leukocytes were separated by hypotonic lysis using red blood cell lysing buffer (Sigma, St. Louis, MO) and Hanks balanced salt solution (HBSS, Gibco, Carlsbad, CA) to restore isotonicity. Tissues were homogenized with a tissue grinder, then total RNA was extracted from blood leukocytes, lung lavage cells, spleen and thymus cells using Rneasy mini kits (Qiagen, Valencia, CA) for extraction. Samples were frozen (−80 °C) in 50 μl of water after quantifying total RNA using 260 and 280 nm readings by spectrophotometer.

Real-time RT-PCR was used to quantify GAPDH, IL-1, IL-1 receptor antagonist (IL-1Ra), TNF-α, toll-like receptor 2 (TLR2), and toll-like receptor 4 (TLR4). Reverse transcription was done with random hexamers in a 100 μl final volume that contained 50 U/μl MultiScribe reverse transcriptase, 25 mm $MgCl_2$, 2.5 μM random hexamers, 0.4 U/μl Rnase inhibitor, 50 μM dNTPs, and TaqMan RT buffer (TaqMan reverse transcription reagents, Applied Biosystems, Foster City, CA). The following mixture was incubated at 25 °C for 10 min, heated to 37 °C for 60 min and inactivated at 95 °C for 5 min. The final volume was stored at −80 °C until used. Real-time TaqMan PCR for the internal control and the genes of interest were run in separate wells. The PCR reactions were placed into a 96-well plate and contained 900 nm of each primer, 250 nm of the TaqMan MGB probe and PCR Mastermix (TaqMan Universal PCR Mastermix, no AmpErase UNG, Applied Biosystems, Foster City, CA) and 5 μl of the diluted cDNA samples in a final volume of 50 μl. The samples and standards were amplified in an automated fluorometer (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Foster City, CA) with an initial incubation of 2 min at 50 °C and then heated for 10 min at 95 °C for activation of the AmpliTac Gold, then 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The final quantitation was done using a standard curve generated by dilutions of the gene of interest or the internal standard (GAPDH). Data are reported as relative transcription of the gene of interest relative to the internal standard. Primers and probes (Table 1) were generated by PrimerExpress software (Applied Biosystems, Foster City, CA) using published sequences of the genes of interest.

2.1. Statistics

Chi-square analysis (SAS, 2002) was used to determine morbidity and mortality differences. Real-time

Table 1
Primers and probes used for real-time RT-PCR

Gene	Primers	Probe	Reference/Accession no.
GAPDH	f AAGGCCATCACCATCTTCCA r CAGCATCACCCCACTTGATGT	AGCGAGATCCTGCC (FAM/MGB)	Taylor et al. (1996)
IL-1β	f TTCCTGTGGCCTTGGGTATC r TGGGCGTATCACCTTTTTTCA	CAAGAATCTATACCTGTCTTGT (VIC/MGB)	Ito and Kodama (1996)
IL-1Ra	f CCTCCTTTCTCACCCCAGATC r AGAAAATGGAAGCCGCTTAGG	CAGGCGCTCACTTC (VIC/MGB)	Kirisawa et al. (1998)
TNF-α	f TGGGAAGCTTACCTTTTCCTTTC r CTTCTTCATGACCCAGATACATCCT	CCTCAAGTAACAAGCCG (VIC/MGB)	Bienhoff and Allen (1995)
TLR2	f CCACGGAAGGAGCCTCTGA r GCCATCGCAGACACCAGTT	CAGGCTTCTTCTCTGTCTT (VIC/MGB)	AF368419
TLR4	f CCGGATCCTAGACTGCAGCTT r TCCTTGGCAAATTCTGTAGTTCTTG	CCGTATCATGGCCTCT (VIC/MGB)	AAG32061

RT-PCR was performed on the samples in duplicate. The standards were performed in triplicate and values were determined by solving for the unknown using linear regression. The R^2 for standard curves were 0.98, 0.93, 0.97, 0.99, 0.97, and 0.99 for GAPDH, IL-1, IL-1Ra. TLR2, TLR4, and TNF-α respectively. Gene quantities were then calculated as a ratio of the gene of interest to the corresponding internal standard for that sample. Data were analyzed as a randomized complete block using the repeated statement in the Mixed Model procedures of SAS (Littell et al., 1996) for the blood leukocyte analysis. Block was used as a fixed effect. Compound symmetry, unstructured, heterogeneous first-order autoregressive, and first-order anti-dependence covariance structures were analyzed for the best fit. Compound symmetry was the best fit for most variables. Tissues were analyzed as a randomized complete block design using the GLM procedure in SAS.

3. Results

The doses of the treatments were based on recommended dosages currently in use. However, the combination treatment resulted in greater morbidity (calves requiring assisted eating, low or negative weight gain, receiving electrolytes or antibiotic treatments) and mortality ($P < 0.05$); 100, 57, 42, and 29% survival for Cnt, GH, DEX, and Both, respectively. The means ± S.D. for GAPDH cycle thresholds were 0.419 ± 0.360, 0.049 ± 0.024, 0.058 ± 0.041, and 0.290 ± 0.250 for blood leukocytes, spleen cells, thymus cells, and lung lavage cells, respectively, and was not different among treatments or days. The blood leukocytes were not sampled until the calves had been treated for 2 weeks because of interference with the ongoing metabolic component of this experiment (Hammon, unpublished data). By day 14, TLR2 was expressed two times less and TLR4 was expressed three times less in blood leukocytes (Fig. 1) by the GH and Both treated calves than for the Cnt calves ($P < 0.05$). On day 28, the DEX treated calves tended ($P < 0.10$) to express more TLR2 in blood leukocytes than the GH calves, but were not different than the Cnt or Both calves. Also, on days 14 and 28, the DEX treated group expressed significantly more IL-1Ra in blood leukocytes than the Cnt, GH, and Both calves ($P < 0.05$), but IL-1 expression was greater ($P < 0.05$) for controls than for the treated calves on day 14 and overall was quite variable throughout the study. The expression of TNF-α in blood leukocytes was greater ($P < 0.05$) for the Cnt calves than for the GH and Both group on day 14, but DEX treated calves tended ($P < 0.10$) to express more TNF than the Cnt and Both groups on day 28. The last two samples taken on days 42 and 56 were not different among treatments for blood leukocyte expression of TNF.

Toll-like receptor expression of bronchial alveolar lavage cells (Fig. 2), was greater ($P < 0.05$) for TLR2 of the Both treated calves than for the DEX or GH treated calves, and tended ($P < 0.10$) to be greater than for the Cnt. However, TLR4 tended ($P < 0.10$) to

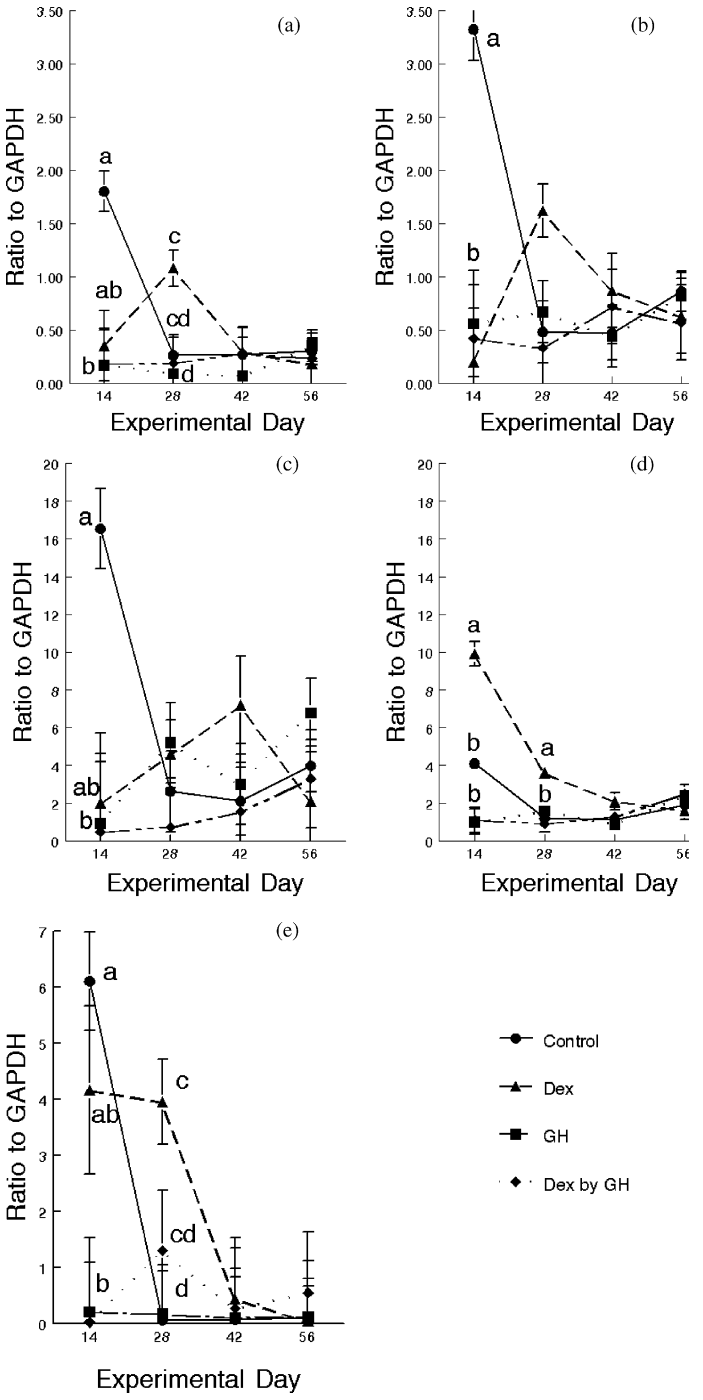

Fig. 1. RNA expression of (a) TLR2, (b) TLR4, (c) IL-1, (d) IL-1Ra, and (e) TNF-α for blood leukocytes from calves that were treated with saline (Cnt), dexamethasone (DEX), growth hormone (GH), or DEX and GH (Both). Data are expressed as a ratio of gene of interest cycle threshold to GAPDH cycle threshold (mean ± S.E.). Means within a day without common superscripts differ: a, b ($P < 0.05$) and c, d ($P < 0.10$).

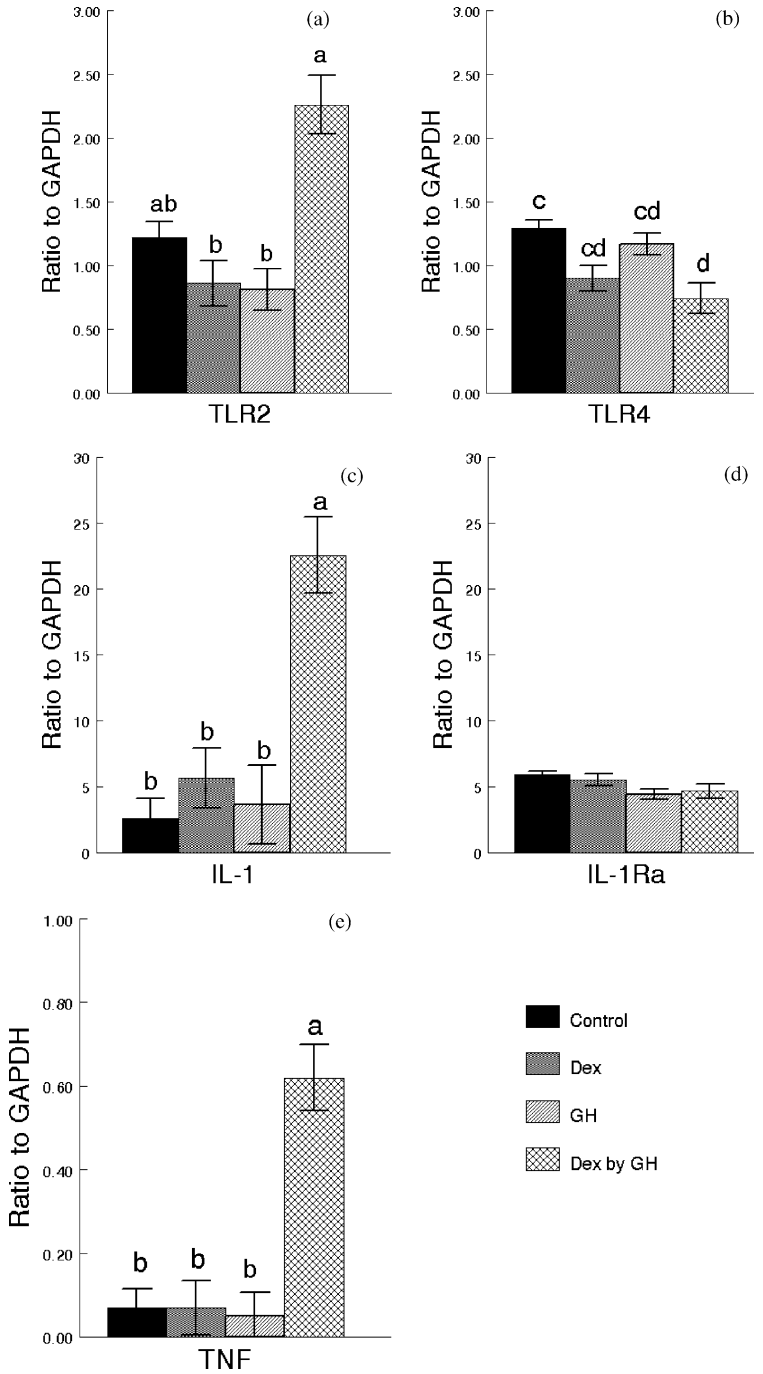

Fig. 2. RNA expression of (a) TLR2, (b) TLR4, (c) IL-1, (d) IL-1Ra, and (e) TNF-α for bronchial alveolar lavage cells from calves that were treated for 56 days with saline (Cnt), dexamethasone (DEX), growth hormone (GH), or DEX and GH (Both). Data are expressed as a ratio of gene of interest cycle threshold to GAPDH cycle threshold (mean ± S.E.). Means within a day without common superscripts differ: a, b ($P < 0.05$) and c, d ($P < 0.10$).

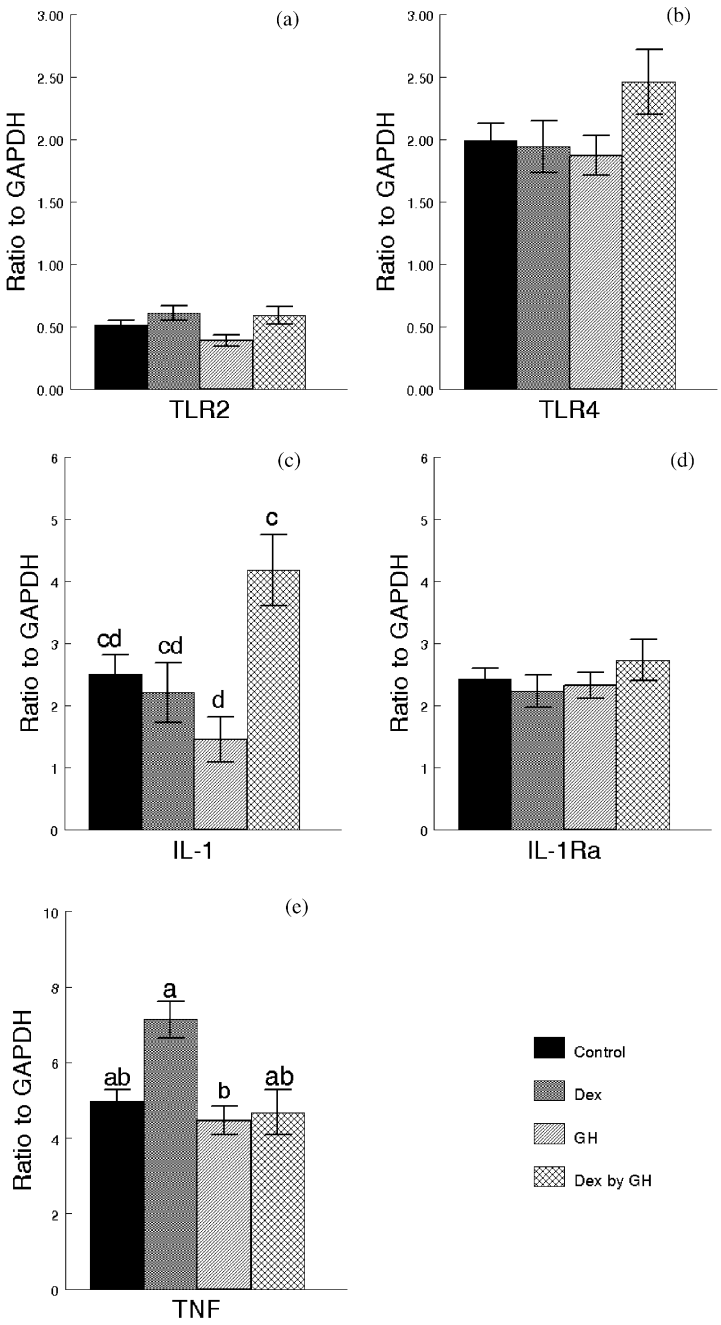

Fig. 3. RNA expression of (a) TLR2, (b) TLR4, (c) IL-1, (d) IL-1Ra, and (e) TNF-α for spleen cells from calves that were treated for 56 days with saline (Cnt), dexamethasone (DEX), growth hormone (GH), or DEX and GH (Both). Data are expressed as a ratio of gene of interest cycle threshold to GAPDH cycle threshold (mean ± S.E.). Means within a day without common superscripts differ: a, b ($P < 0.05$) and c, d ($P < 0.10$).

be reduced in Both treated calves compared to the Cnt calves. Interleukin-1 expression of bronchial alveolar lavage cells was increased nearly four-fold ($P < 0.05$) by the Both treatment compared to the other three treatments. In contrast no differences were observed among treatments for IL-1 receptor antagonist expression. Tumor necrosis factor-α expression of bronchial alveolar lavage cells was greatly enhanced (approxi-

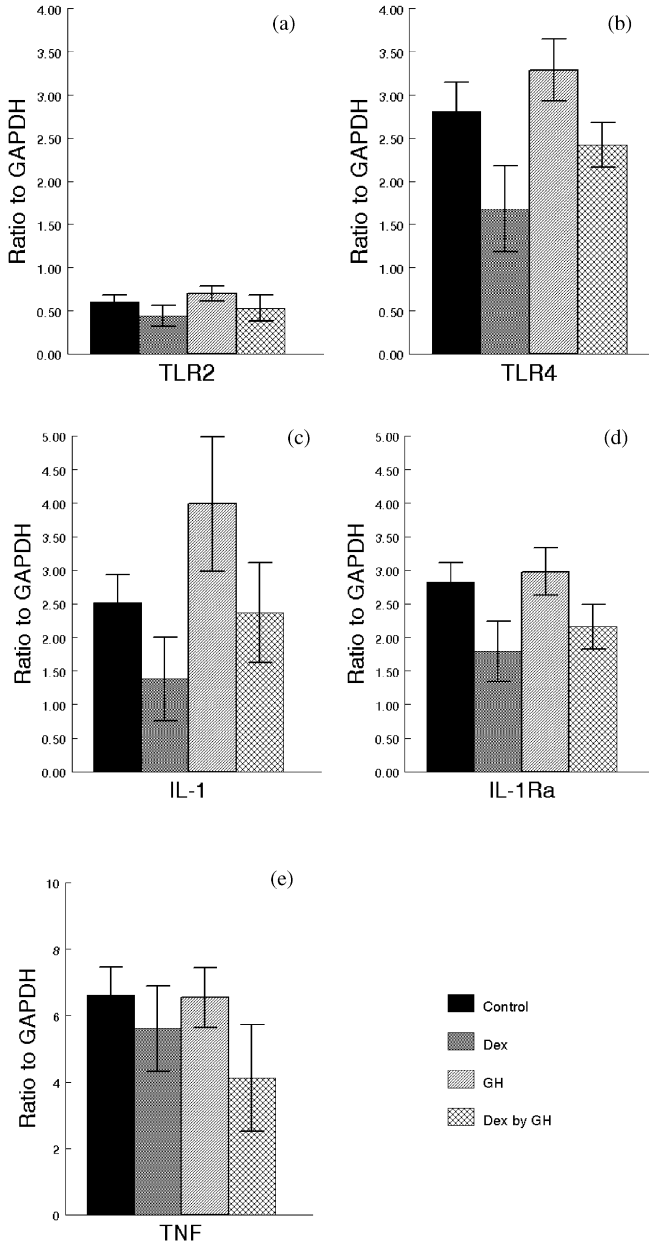

Fig. 4. RNA expression of (a) TLR2, (b) TLR4, (c) IL-1, (d) IL-1Ra, and (e) TNF-α for thymus cells from calves that were treated for 56 days with saline (Cnt), dexamethasone (DEX), growth hormone (GH), or DEX and GH (Both). Data are expressed as a ratio of gene of interest cycle threshold to GAPDH cycle threshold (mean ± S.E.).

mately six times) by the Both treatment compared to the other three groups ($P < 0.05$).

No differences in TLR2 or TLR4 expression were detected in the spleen tissues (Fig. 3). Interleukin-1 expression tended ($P < 0.10$) to be greater in the spleen for the Both treated calves compared to the GH only treated calves. No differences were seen among the treatments for IL-1Ra expression in the spleen cells. The spleen cells of DEX treated and GH treated calves were different in expression of TNF-α. Dexamethasone treatment resulted in greater TNF-α expression in spleen cells ($P < 0.05$) compared to the TNF-α expression in spleen cells of GH treated calves, and dexamethasone tended ($P < 0.10$) to cause greater TNF-α expression compared to the TNF-α expression in spleen cells of the Cnt calves.

Thymus cells (Fig. 4) were not different in expression of TLR2 or TLR4. The TLR4 expression had greater variability than did the TLR2 expression. The thymus cells also had greater variability in IL-1 and IL-1Ra expression than the other tissues, but were not different among the treatments. Nor were differences in TNF-α expression detected among the thymus cells.

4. Discussion

The lower TLR2 and TLR4 expression, and reduced expression of IL-1 by blood leukocytes of DEX, GH and DEX by GH calves compared to control calves' leukocytes by day 14 together show how calves at 2–4 weeks of age are more vulnerable to disease. We were unable to get day 3 blood leukocyte data in this study, but in other work from our laboratory the expression TLR2 and TLR4 in blood leukocytes from 3-day-old calves was similar to the DEX treated calves at day 14. This finding is partially supported by Diez-Frainle et al. (2000) who showed suppression of CD11b on bovine neutrophils with dexamethasone. This early neonatal time period is when maternal antibody concentrations are declining (Husband et al., 1972). With the addition of growth hormone or glucocorticoids, the effect appears to be pronounced and affecting the ability of the cells to express TLR2 and TLR4, which are essential in initiating both innate and specific immune responses. It follows that within 2–4 weeks, that these calves whose passive protection is waning and are unable to initiate their own immune response, would become morbid and could have increased mortality. Since all but one of the deaths occurred between experimental days 42 and 56, it is surmised that they were the result of chronic suppression by the DEX by GH treatment. The DEX by GH treatment was the only treatment that had little change in IL-1 expression. The GH only calves showed a slight IL-1 expression change at day 28 and again at day 56. Both treatments containing GH did not change expression of TLR2, TLR4 or IL-1 receptor antagonist. This suggests that the ability to initiate the IL-1 response was essential to protect the calf from normal environmental pathogens.

Recent data have shown a synergistic effect of glucocorticoids on TLR2 expression with *H. influenzae* stimulation (Shuto et al., 2002). This research showed that the two known pathways of TLR2 activation, NF-κB and MKK3/6-p38α/β did not have to be activated to attain TLR2 up-regulation. Other research showed that growth hormone, initiates a survival signal through activation of the NF-κB pathway (Jeay et al., 2000), but TNF-α protein concentrations were not affected by the growth hormone, bovine somatotropin (Cappuco et al., 2001). We saw both increased TNF-α and IL-1 expression by bovine lung cells when GH and DEX were delivered together. Santos et al. (2001) showed that specific tissues respond differently to macrophage migration inhibitory factor in the presence of glucocorticoids. Molecules that are involved in the regulation (CD14) of toll-like receptors are tissue specific as well. Skin and pancreas tissues increase P-selectin (a molecule important in cell adhesion), but not lung or muscle in response to LPS of CD14 deficient mice. Sequestering of leukocytes to some tissues is CD14 and TLR4 dependent, but others are CD14 independent and TLR4 dependent, further demonstrating tissue specific responses (Andonegui et al., 2002). Our data were very tissue specific. This is interesting because of having only the end point analysis of the pulmonary alveolar lavage cells, we do not know if our results are a direct effect of the treatments on lung immune cells or if the enhanced TLR2 expression was in response to a specific pathogen because of suppressed immunity.

The interaction of growth hormone and glucocorticoids was beneficial for children with inflammatory bowel disease (Mauras et al., 2002). Body composition changes and growth were improved with the

simultaneous administration of both hormones. However, no cytokine or toll-like receptor data was included within this study. So, the effects of these hormones on more discrete measures such as TLR expression or activation of pathways leading to immunity are not known.

Little of published research is available regarding toll-like receptors in cattle. Recent research has explored the recognition of bacterial components on TLR2, TLR4 and CD14 expression (Guionaud, unpublished data). Additionally, TLR2 and TLR4 were detected in monocytes, macrophages, and dendritic cells, but no differences in mRNA transcript quantity were detected between these two TLR (Werling and Jungi, 2003). Dendritic cells were also reported to react differently than monocytes and macrophages to typical TLR2 and TLR4 ligands. Other research has explored the related cytokines (Burton et al., 2001; Leutenegger et al., 2000). But much is unknown about the function of these receptors in applied conditions.

Numerous questions remain unanswered. The species specificity of the function of the toll-like receptors is not known, including the relevance of the multiple pathways. The presence and concentrations of growth hormone and glucocorticoids in neonatal cattle is complicated by the abundance of both of these hormones at birth. So, it is possible that additional hormones over-stimulated these young animals and may have induced many inhibitory effects, including the immune system development and glucose metabolism, thus leaving the calves vulnerable to infection.

5. Conclusion

From these data we can conclude that both dexamethasone and growth hormone act to modulate the immune system, and they work synergistically to enhance RNA expression for TLR2 in the lung leukocyte population.

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